# On the Reactivity of Native Phytochrome

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Previous phytochrome models were based on experimental results obtained only with proteolytically degraded phytochrome. The reactivity of native phytochrome, in both the Pr and the Pfr form, towards 8-anilinonaphthalene-1-sulfonate (ANS), urea, and ethylene glycol is described here. Whereas native phytochrome in the Pfr form is only very slowly bleached by ANS, immediate bleaching occurs with ANS treated Pr during red irradiation. The bleached form, $P_{bl}^{640}$, can be converted into $Pr^{660}$ by prolonged far-red irradiation. A tentative model for native phytochrome is proposed which explains the effects of phototransformation, ANS binding and partial proteolysis.

## Introduction

Phytochrome is the photoreceptor for red-light-mediated morphoses in plants. It is generally accepted that the physiologically active form, Pfr, binds to a presumed receptor [1] and in this form exerts the physiological reactions.

$$Pr \underset{730\,nm}{\overset{660\,nm}{\rightleftharpoons}} Pfr \xrightarrow{X} Pfr\,X \rightarrow \text{physiological response}\,.$$

Differences in the reactivity of Pr and Pfr have been investigated as a model for the presumed receptor binding reaction of Pfr. A model in which a hydrophobic site of the protein is exposed in Pfr was developed by binding studies with 8-anilinonaphthalene-1-sulfonate (ANS) [2]. This site was assumed to be covered in Pr by the chromophore [2]. The chromophore itself should be exposed to the medium only in the Pfr form as deduced from its easy oxidation by permanganate [3]. These results were, however, obtained with phytochrome (114/118 kdalton) which had undergone limited proteolysis [4, 5]. In native phytochrome (124 kdalton), no difference of chromophore oxidation between Pr and Pfr was detected [6]. It is, therefore, questionable whether an exposed hydrophobic site can be detected in the Pfr form of native phytochrome. One parameter of ANS binding to this exposed hydrophobic site is the disappearance of the typical absorption band of Pfr ($\varepsilon^{730\,nm}$ = 65 – 80 000, [7]) and the appearance of a broad absorption band centered at 640 nm ($\varepsilon^{640\,nm}$ = 18 000 [8]). We describe here the investigation of spectral changes of native phytochrome induced by ANS.

*Abbreviations:* ANS = 8-anilinonaphthalene-1-sulfonate, EDTA = ethylenediaminotetraacetate.

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## Materials and Methods

Oat grains (*Avena sativa* L., CV. Pirol, Baywa München) were germinated for 3.5 days at 27 °C in darkness on moist vermiculite. The shoots were harvested under dim-green light [9]. Extraction of phytochrome with Tris-HCl/50% ethylene glycol and purification by poly(ethylenimine) precipitation and chromatography on hydroxylapatite was performed as described by Vierstra and Quail [10]. Elution from hydroxylapatite was completed by 50 mM (instead of 20 mM) potassium phosphate buffer. The subsequent Affi-Gel Blue procedure was omitted. Precipitation with ammonium sulfate and chromatography on Bio-Gel A-1.5 M [10] yielded native phytochrome which was sufficiently pure for spectral studies in the visible range. SDS gel electrophoresis [10, 11] showed only one protein band at about 125 kdalton. Identical spectra were obtained with a sample of pure native phytochrome which was spectrally pure also in the UV range. This sample was kindly provided by Dr. P.-S. Song, Lubbock/Texas.

ANS was recrystallized three times from saturated aqueous $MgCl_2$ before use.

Spectra were recorded on a DMR-22 spectrophotometer (Zeiss, Oberkochen) which was equipped with an irradiation device. Red irradiation was at 660.3 nm with a fluence rate of 64 $Wm^{-2}$; far-red

irradiation was at 728.3 nm with a fluence rate of 31 $Wm^{-2}$.

## Results and Discussion

The effect of 2 mM ANS on the absorption of Pr is shown in Fig. 1 a. In accordance with Hahn and Song [2], we find an apparent blue shift of the absorption band from 666 nm to 663 nm (immediately after addition of ANS) or to 660 nm (after 14 h incubation with ANS). The absorption decreases to about 89% of the original value immediately after the addition of ANS and to about 80% after 14 h incubation with ANS. The reactivity of native Pr (124 kdalton) seems to be identical with or similar to partially degraded Pr (114/118 kdalton).

The effect of 2 mM ANS on the absorption of native Pfr is shown in Fig. 1 b. The absorption peak at 730 nm remains but decreases in its absorption to about 80–85% immediately after the addition of ANS and to about 65% after 18 h incubation with ANS. It appears from these data that the reactivity of Pr and Pfr towards ANS in native phytochrome is similar when monitored by changes of the chromophore absorption. Native Pfr bleaches somewhat more than native Pr. This behaviour is quite different from that of degraded phytochrome in which the Pfr chromophore is completely bleached by ANS under the same conditions [2, 8].

Strong bleaching of native phytochrome (124 kdalton) can be obtained, however, by red irradiation in the presence of ANS. A precondition for effective bleaching is the previous formation of Pr absorbing at 660 nm ($Pr^{660}$). This form can be obtained either by prolonged incubation of native Pr with ANS in the dark (Fig. 1 a) or immediately by far-red irradiation of native Pfr in the presence of ANS. Bleaching of $Pr^{660}$ by red irradiation is shown in Fig. 2. Taken into account the molar extinction coefficients of the phytochrome species in question ($\varepsilon$ values for $Pr^{666}$ (native) = 110 000, for $Pr^{660}$ = 94 000, for $P_{bl}^{640}$ (ANS) = 18 000, see [8]) it can be calculated that 60–65% of total phytochrome are transformed into $P_{bl}^{640}$ by saturating red irradiation. About 15–20% Pfr and 4–5% $Pr^{660}$ are formed under these conditions. Some phytochrome (15–20% in most experiments) is irreversibly lost during irradiation in the presence of ANS.

The behaviour of Pfr in the presence of ANS is different from that of Pr. As outlined in Fig. 1 b,

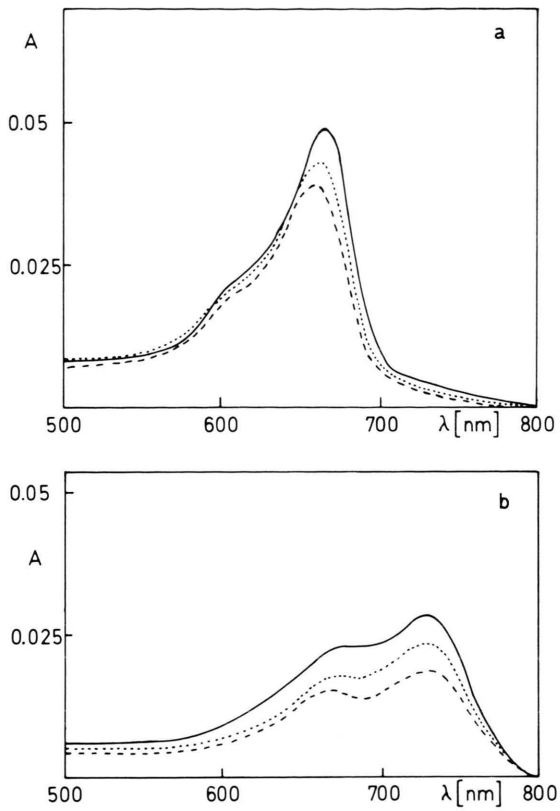


Fig. 1. Effect of ANS onto the absorption spectrum of native phytochrome. (——) phytochrome in 10 mM potassium phosphate buffer pH 7.8, containing 2.8 mM mercaptoethanol and 5 mM EDTA, (. . . .) immediately after addition of ANS in the same buffer, final concentration 2.1 mM, (- - - -) the same solution after incubation with ANS at 4 °C in the dark. a) Pr form, incubation time 14 h, formation of $Pr^{660}$; b) Pfr form, incubation time 18 h.

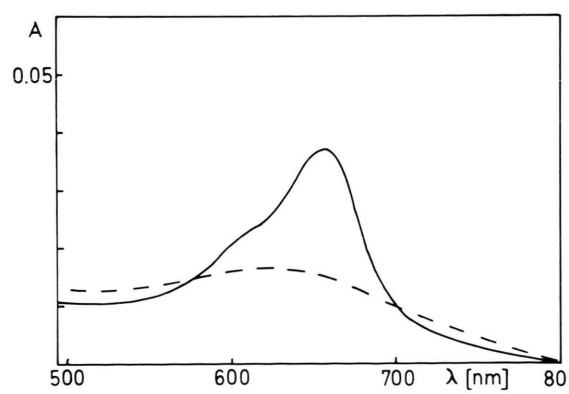


Fig. 2. Effect of red irradiation of $Pr^{660}$ (see Fig. 1 a). Native phytochrome, Pr form, was preincubated in the dark with 3.5 mM ANS for 1 h. (——) formation of $Pr^{660}$, and then irradiated for 1 min with red light (660 nm, 64 $Wm^{-2}$); (- - - -) formation of $P_{bl}^{640}$.

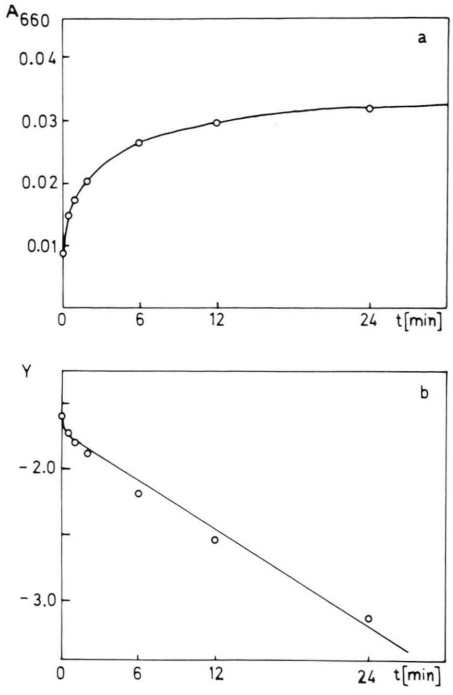


Fig. 3. Kinetics of formation of $Pr^{660}$ from $P_{bl}^{640}$ by continuous far-red irradiation (728 nm, 31 $Wm^{-2}$). The absorbance of the original $Pr^{660}$ was 0.042. 70% of this absorbance (= 0.030) was recovered by saturating far-red irradiation of $P_{bl}^{640}$. a) Increase of $A_{660}$ during far-red irradiation; b) Linearized plot in which $y$ equals lg $(0.030 - A_{660})$. The fast kinetic component (0 to 0.5 min) belongs to the transformation of residual Pfr to $Pr^{660}$.

we observe some irreversible bleaching which could be due to formation of some $P_{bl}^{640}$. The main absorption band of Pfr remains, however, at 730 nm. Far-red irradiation yields $Pr^{660}$ in stoichiometric amounts. This is the fastest and most effective method of $Pr^{660}$ formation, much faster than the incubation of $Pr^{666}$ with ANS in the dark.

The bleached form $P_{bl}^{640}$ can still be phototransformed into $Pr^{660}$ by far-red irradiation. Because the absorbance of this species at 730 nm is very low, long periods of irradiation are needed (Fig. 3). A new photoequilibrium between $Pr^{660}$ and $P_{bl}^{640}$ is established by this irradiation. Because the transformation of $Pr^{660}$ to $P_{bl}^{640}$ does not need as much light as the reversion, white light leads to preferential formation of $P_{bl}^{640}$.

The results can be summarized in a tentative model (Fig. 4) which also takes into account the relevant data from the literature. In the Pr form, interaction of the chromophore with specific sites of the protein leads to the extended conformation of phytochromobilin which is characteristic for native Pr [12]. A hydrophobic site (H in Fig. 4) which is a binding site for ANS is nearly covered by the protein. ANS can only slowly bind to this site. This binding can be monitored by the absorption shift from 666 to 660 nm. In the Pfr form, interaction of the chromophore with specific sites of the protein leads to the 730 nm absorption band. The chromophore binding sites of Pfr which are different from the binding sites in Pr probably involve the ANS binding site. If this site is occupied by ANS, the specific interaction with the chromophore is interrupted so that no 730 nm absorption appears. A bleached form of phytochrome ($P_{bl}^{640}$) appears instead for which our model assumes neither Pr nor Pfr specific chromophore-protein interactions. Bleached forms of phytochrome can be produced chemically but are also natural intermediates of the Pr → Pfr phototransformation (see [8]). The prediction of the cyclic conformation in such bleached forms like in protein-free bilins [13] agrees with our view of lack of specific interactions with the protein. Our model furthermore explains the stability of Pfr (124 kdalton) against the attack of ANS because the binding site is not accessible for ANS.

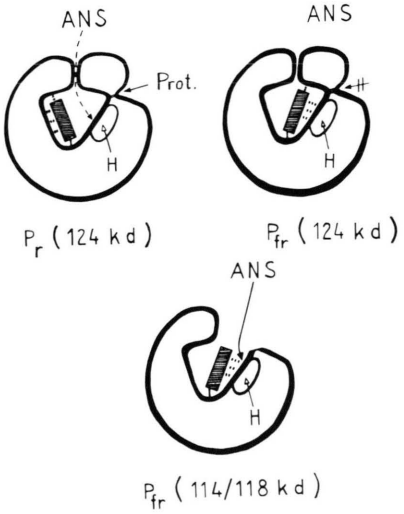


Fig. 4. Proposed spatial model for native Pr, Pfr and partially degraded Pfr. This model explains the differential reactivity of the different phytochrome forms with ANS. H = hydrophobic site, Prot = site of proteolytic attack. This proteolysis only occurs in the Pr form. Shadowed rectangular = chromophor. Interactions of the protein with the chromophor are indicated by dotted lines.

The binding site is, according to our results, more accessible *during* the phototransformation from Pfr to Pr; the $Pr^{660}$ species is therefore formed immediately after far-red irradiation of Pfr in the presence of ANS.

Partial proteolysis occurs with Pr (124 kdalton) to Pr (114/118 kdalton) but not with Pfr (124 kdalton) because the cleavage site is somehow protected in the Pfr form. This has been explained by interaction of the extra segment of the native protein with the chromophore in the Pfr form [14] as indicated in Fig. 4. The Pfr form (114/118 kdalton) has less interaction between chromophore and protein; hence the chromophore absorption is shifted to only 720–725 nm. Its hydrophobic site is more exposed than in Pr [15] and therefore more easily accessible for ANS [2]. This Pfr form is therefore immediately bleached by ANS and other chemicals [8].

The model also explains why further proteolysis yields phytochrome forms (40, 39, and 33 kdaltons) which exhibit reversible conversion between $Pr^{660}$ and $P_{bl}^{640}$ [16]. In this case, the Pfr specific interactions probably were removed by proteolysis which gives the same spectral effect as occupation of the hydrophobic site by ANS. Further experiments are needed to refine the model for native phytochrome, *e.g.* looking for photoreversible pH changes like in degraded phytochrome [17] and for intermediate spectral forms [7].

We have also investigated the influence of urea onto the spectrum of phytochrome (124 kdalton). As with Pr (114/118 kdalton) [8], urea concentrations up to 4.8 M produce $Pr^{660}$, *i.e.* the absorption of Pr (124 kdalton) is only slightly shifted from 666 to 660 nm with a decrease in extinction of about 10–15%. Red irradiation of this $Pr^{660}$ species immediately leads to bleached phytochrome. Contrary to the results with ANS, native Pfr (124 kdalton) is also bleached by addition of urea in the corresponding concentration. This means that the attack of urea is different from that of ANS although the spectral effects seem to be very similar. On the other hand, native Pr and Pfr are stable towards ethylene glycol (up to 50%) whereas Pfr (114/118 kdalton) is immediately bleached completely by 20% ethylene glycol [8].

*Acknowledgements*

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